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The Effect of Sulfur Fertilizer on Glucoraphanin Levels in Broccoli (*B. oleracea* L. var. *italica*) at Different Growth Stages

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Three sulfur (S) treatements were imposed by applying gypsum to three broccoli cultivars (Claudia, Marathon, and TB-234) known to differ in glucoraphanin content of mature seeds. The S treatments were control (very low added S), low S (23 kg S ha^{-1}), and high S (92 kg S ha^{-1}). The gypsum applications during the early vegetative phase of the three broccoli cultivars increased S uptake and the glucoraphanin content in each plant organ. There were significant genotypic differences for the content of both S and glucoraphanin in all plant organs at different growth stages with gypsum applications. A large increase in S and glucoraphanin content was found in the green heads of broccoli and mature seeds. S present in glucoraphanin accounted for only 4−10% of total S content in broccoli heads. However, S present in glucoraphanin in mature seeds accounted for 40−46% of the total S in the seeds of moderate and high glucoraphanin cultivars (Marathon and TB-234). The partitioning of S into glucoraphanin also increased with gypsum applications. Differences in S uptake, S distribution between organs, and partitioning of S into glucoraphanin largely explained the differences in glucoraphanin content in the green heads and mature seeds for the three broccoli cultivars and three S treatments.

KEYWORDS: Glucoraphanin; broccoli; gypsum; sulfur

INTRODUCTION

Cruciferous vegetables especially cabbage, cauliflower, mustard, and broccoli have been shown to possess anticarcinogenic activity (*1*−*3*). They contain substantial quantities of the health-promoting phytochemical compounds, including flavonoids, phenolic compounds, and glucosinolates (*4*, *5*). Chopping or chewing of cruciferous vegetables releases the enzyme myrosinase (thioglucoside glucohydrolase, EC 3:2:3:1), which initiates rapid hydrolysis of glucosinolates to yield isothiocyanates, thiocyanates, and other minor metabolites (*6*). The previous studies showed that isothiocyanates, such as allyl isothiocyanate, phenyl isothiocyanate, and sulforaphane, potentially contributed to the cancer prevention of these cruciferous vegetables (*3*, *7*−*9*).

Aliphatic glucosinolates such as glucoraphanin are derived from the sulfur-containing amino acid L-methionine (*10*, *11*). Several reports have indicated that S supply affects the glucosinolate content and/or composition in *Brassica* species (*12*−*17*). The balance between nitrogen and S supply also had an important role in the regulation of glucosinolate synthesis especially for alkenyl glucosinolate synthesis (*16*, *18*). S applied as gypsum or other forms (potassium sulfate, ammonium sulfate, or magnesium sulfate) was shown to increase glucosinolate content in vegetative tissues, flowers, pods, and seeds of *Brassica napus* (*14*, *15*, *17*, *19*, *20*). In addition, S application to S deficient soils resulted in a larger response in the alkenyl glucosinolates than in the indole glucosinolates since the S-containing amino acid, L-methionine, was required for alkenyl glucosinolate biosynthesis (*15*, *16*).

Brassica oleracea var. *italica* (broccoli) was found to contain high levels of glucoraphanin, a glucosinolate precursor of the isothiocyanate sulforaphane, which has been shown to be a potent inducer of phase II enzymes in the body to detoxify cancer-causing chemicals (*2*, *21*). There is minimal information on the effect of S nutrients applied as soil fertilizers on glucoraphanin levels in broccoli during plant development. Recently, Vallejo et al. (*5*) examined the effect of sulfur fertilizer on glucosinolates in three broccoli cultivars at different stages of inflorescence development. Previous studies on the distribution of glucoraphanin in broccoli have indicated that there were

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large differences in glucoraphanin levels among broccoli cultivars and between plant organs and that changes in glucoraphanin levels occurred throughout plant development (*22*, *23*). Therefore, the objective of this study was to examine the effect of S applied as a soil fertilizer (gypsum) on sulfur and glucoraphanin accumulation in different plant organs of broccoli from the vegetative stage to maturity. The partitioning of S and glucoraphanin within the plant and partitioning of S into glucoraphanin were also examined in this study.

MATERIALS AND METHODS

Plant Material. Seeds of broccoli cultivars Claudia, Marathon, and TB-234 were obtained from Henderson Seeds Pty. Ltd. (Victoria, Australia). The specific cultivars were selected because these were known to have low (Claudia), moderate (Marathon), and high (TB-234) concentrations of glucoraphanin in mature seeds (*23*). Seeds were sown on 24 June, 2000, in trays and grown in a nursery until transplanting on 8 August, 2000.

Field Experiment. The field trial was located at Henderson Seeds Pty. Ltd. (latitude 37° 46′ S and longitude 145° 06′ E). The plots were established on a sandy clay loam soil (pH 6.0 at the top 10 cm of soil profile). Average temperatures (min−max) for winter (June−August), spring (September−November), and early summer (December−January) were 5.7−14.1, 10.2−19.5, and 11.3−23.3 °C, respectively, and average rainfalls were 50.6, 83.5, and 23.3 mm, respectively. The experiment was established on raised beds (two rows per raised bed) and was designed as a split plot with three replicates. The main plots were three S treatments (control, low S, and high S) with subplots of the three broccoli cultivars (Claudia, Marathon, and TB-234). The size of each plot was 1.5 m wide and 5.0 m long (total 27 plots). There were two rows in each plot, which contained 10 plants per row.

Young broccoli plants were transplanted to the field on 8 August, 2000. The plots received a base fertilizer dressing of Pivot (N:P:K:S) (8:11:10:7) (S as sulfate) at a rate of 1 kg ha^{-1}. Additional S was applied as gypsum (anhydrous calcium sulfate, which contained 23% S) by hand on the top of the soil in each row on the same day as transplanting. Gypsum was applied at rates of 50 kg ha^{-1} for low S treatment (11.5 kg of S ha^{-1}), 200 kg ha^{-1} for high S treatment (46 kg of S ha^{-1}), and no additional gypsum in control treatment plots. Six weeks after transplanting, Roundoff (N:P:K) (21:0:16) was applied to the field plots at a rate of 100 kg ha^{-1}. Regent, Nitofol, and Pirimor were used for insect control during the trial. All chemicals were supplied by Muir E. E. and Son, Pty. Ltd. (Australia). Gypsum was applied to the soil (topdressing) for the second time, 7 weeks after transplanting (after the vegetative stage harvest) at the same rates as at transplanting. Total S applied to the soil of control plots was only 70 g ha^{-1} (from base fertilizer), low S plots 23 kg ha^{-1}, and high S plots 92 kg ha^{-1}. Soil samples (top 10 cm depth) were also taken from the field before and after S application (each plot treatment with three replicates) to determine S content in the soil and pH.

Sampling Times and Tissues. Glucoraphanin was extracted from plants of broccoli cultivars at four developmental stages: (i) seedling stage (transplanting, 60 days after sowing DAS), (ii) vegetative stage (88 DAS), (iii) green head (green floret, 10−15 cm at 108 to 125 DAS), and (iv) maturity (220 to 235 DAS).

Plants were separated into different parts at each sampling: (i) seedling stage: leaves, shoots, and roots; (ii) vegetative stage: leaves, stems and leaf petioles, and roots; (iii) green head stage: leaves, stems and leaf petioles, roots, and green heads; and (iv) maturity: stems, roots, pods, and seeds.

Two plants (one from each row) were harvested from each plot. Each plant was cut into symmetrical halves from the top (head) to the bottom (root), and one-half from each plant was taken and bulked together as one replicate. The other half was used to determine dry matter of plant organs at the same developmental stage and also for determination of S content in plant organs.

Preparation of Plant Extracts. *Vegetative Tissues.* Plant samples were weighed, separated into stems, petioles, and leaves, and immediately placed in a freezer at −20 °C until extraction the next day. Plant material was cut into pieces of 4−5 cm length after removal from the freezer and immediately plunged into boiling water (700 mL for leaves, 500 mL for stems and petioles and broccoli florets, and 200 mL for roots and pods) for 3 min. Fifty pods per replicate were used for extraction after seed removal. The samples were then extracted for glucoraphanin as previously described (*22*). After the samples were boiled for 3 min, plant materials were homogenized with a blender and then placed on a shaker for 10 min at room temperature. The plant residues were re-extracted twice with 120 mL of boiling water for each extraction. All extracts were combined together and concentrated to 50 (leaves) and 40 mL (other plant materials) at 40 °C. All concentrated extracts were centrifuged at 2000*g* for 10 min and vacuum-filtered through filter paper (Whatman No. 1) into volumetric flasks. The volumes were adjusted with distilled water to 100 and 50 mL for leaf and other extracts, respectively.

Seeds. Glucoraphanin was extracted from seeds (30 seeds per replicate) at maturity as previously described (*23*). Boiling water (6.0 mL) was added to approximately 0.2−0.4 g of seeds and maintained at boiling temperature for 3 min. Samples were then homogenized with a mortar and pestle and placed on a shaker for 10 min. The residues were re-extracted twice with 5.0 mL of boiling water for each extraction. The volume was then adjusted to 20 mL with distilled water. All samples were filtered through a 0.2 μm aqueous membrane. Extracts were stored at −15 °C.

Reverse Phase Paired Ion Chromatography of Glucoraphanin. The level of glucoraphanin was determined as previously described (*22*) on an analytical μ-Bondapak C_{18} reverse phase column (10 μm, 3.9 mm × 300 mm) connected to μ-Bondapak C_{18} Guard-pak (Water, Melford, MA) with a detection at 230 nm (flow rate, 1.0 mL min^{-1}). To determine fresh weight/dry weight ratios, plant materials from each sampling time were weighed, dried at 80 °C for 48 h, and then reweighed.

Sulfur Analysis. *Acid Digestion.* Acid digestion was carried out following the method of Zarcinas et al. (*24*) with some modifications. Dry plant tissues were ground until they were fine enough to pass through a 1 mm stainless steel sieve using a Makla mill (Crompton Parkinson Pty. Ltd., Australia). Nitric acid (5.0 mL) was added to 0.5 g of dried plant sample (0.15 g for seed sample) in a 100 mL digestion tube and spun at low speed to mix the plant tissues with the acid. Sample tubes were then placed into the digestion block (Kjeldatherm) and heated to 90 °C for 45 min (tubes were swirled if frothing occurred). Small funnels were placed on the top of the tubes to avoid sample loss when frothing occurred. The temperature was then increased slowly to 140 °C, and digestion continued at this temperature until about 2 mL of acid remained (acid solution was clear). After they were cooled, the digests were diluted to 20 mL with 1% nitric acid in 50 mL tubes, mixed, and centrifuged at 2100*g* for 5 min. For leaf tissues, 2.0 mL of acid solution was transferred into 20 mL volumetric flasks and the volume was adjusted with 1% nitric acid. For stems, roots, and seed samples, 1.0 mL aliquots of the acid solutions were transferred into 50 mL volumetric flasks. For broccoli head and pod samples, 1.0 mL aliquots of the acid solutions were transferred into 100 mL volumetric flasks. After dilution, all samples were kept at 4 °C until analysis.

Inductively Coupled Plasma Spectrometry (ICPS) Analysis. S content in the digested samples was measured by ICPS analysis using a Perkin-Elmer Optima 300 (U.S.A.) with a Hg lamp. Digest solutions were introduced into the plasma using a nebulizer at the rate 0.8 L min^{-1}. The plasma flow rate was 15 L min^{-1}, and the auxiliary rate was at 0.5 L min^{-1}. A wet plasma aerosol type was used, and the pump sample flow rate was 2.0 mL min^{-1} with a flushing time of 20 s. The reading time was 20 s with peak area integration. S was detected at 180.669, 182.563, and 189.965 nm. The most sensitive line for S was 180.669 nm; thus, the data from this wavelength were used for the S analysis. S content was detected in the ppm range (μg mL^{-1}) with three readings taken for each sample.

Statistical Analysis. Analysis of variance was carried out using General Linear Model for glucoraphanin concentration, content, and S content data for the three S treatments and the three broccoli cultivars. Mean separation was determined by least significant differences (Fisher's LSD) at $P = 0.05$.

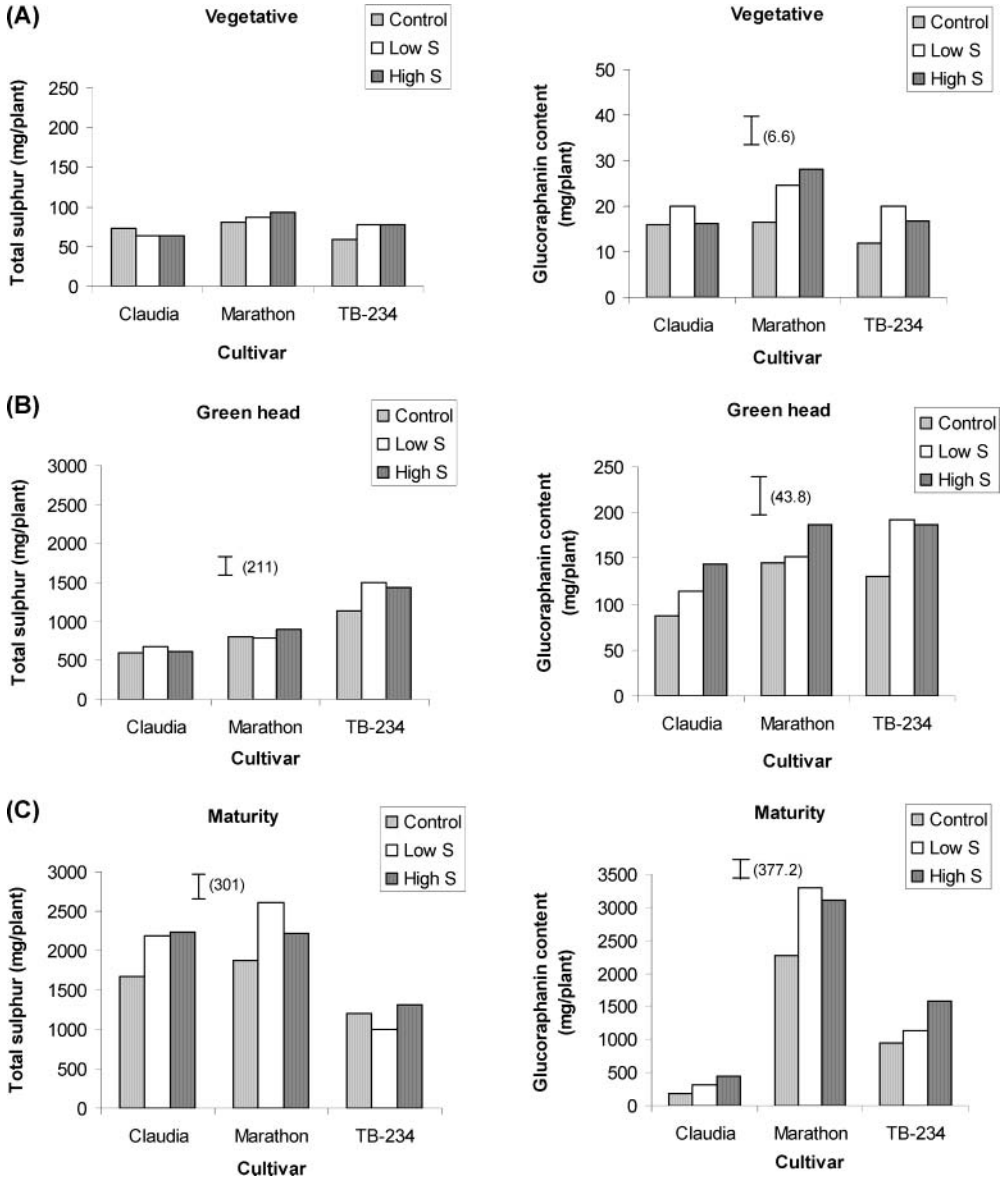

Figure 1. Plant S content (S uptake) and glucoraphanin content of three broccoli cultivars (Claudia, Marathon, and TB-234) at different growth stages (**A**: vegetative, 88 DAS; **B**: green head, 108 or 125 DAS; and **C**: maturity, 220 or 235 DAS). Vertical bars indicate the least significant difference (df = 16) of mean for CVxS comparisons.

Table 1. Total Dry Matter (g) of the Whole Plant as Affected by S Supply in Three Broccoli Cultivars (Claudia, Marathon, and TB-234) at Different Growth Stages (Vegetative, 88 DAS; Green Head, 108 or 125 DAS; and Maturity, 220 or 235 DAS)[a]

	vegetative			green head			maturity		
stage/cultivar	C	LS	HS	C	LS	HS	C	LS	HS
Claudia	14.5	12.7	12.5	72.1	80.6	74.0	160.7	180.2	187.5
Marathon	12.0	13.1	14.1	92.7	86.5	94.9	160.5	214.3	187.8
TB-234	8.8	11.2	10.8	113.0	140.9	137.6	150.4	125.6	163.8
LSD (CV or S)		1.8			13.7			15.7	
LSD (CVxS)					23.8			27.2	
Sig CV		**			***			***	
S		NS			NS			*	
CVxS		NS			*			**	

[a] Probability level: ***$P < 0.001$, **$0.001 < P < 0.01$, *$P < 0.05$, NS = not significant, 16 degrees of freedom for LSD. C = control, LS = low S, and HS = high S.

RESULTS AND DISCUSSION

Soil Analysis. Before transplanting, the soil pH was on average 6.0 ± 0.02 (SE) and at the end of the trial had changed little (pH 6.1 ± 0.01 in control plots, 6.0 ± 0.03 in the low S plots, and 5.9 ± 0.01 in the high S plots). In control plots, soil S concentration significantly decreased from the beginning to the end of the trial, as a result of S uptake by broccoli plants. S concentration in the low S plots was 1.2 times higher than the control plots (297 mg kg^{-1}) and in the high S was 1.8 times higher than the control plots and 1.5 times higher than low S plots at the end of the field trial.

Dry Matter Accumulation. Gypsum applications significantly increased plant dry matter at the green head stage for TB-234 and at maturity for Marathon (**Table 1**). In canola, Hocking et al. (*20*) found that there were no significant differences in shoot dry matter between low and high S (applied as potassium sulfate) until the start of stem elongation; top-dressing with high S at stem elongation increased anthesis biomass (*20*).

There were significant differences in plant dry matter between the three broccoli cultivars at all growth stages (**Table 1**). Claudia had the highest dry matter at the vegetative stage while dry matter of TB-234 was the highest at the green head stage.

Table 2. Effects of S Supply on S (A) and Glucoraphanin (B) Contents (mg Organ^{-1}) in Leaves, Stems and Leaf Petioles, and Roots of Broccoli Cultivars Claudia, Marathon, and TB-234 at the Vegetative Stage (88 DAS)[a]

	leaves			stems and leaf petioles			roots		
organ/cultivar	C	LS	HS	C	LS	HS	C	LS	HS
				A					
Claudia	41.9	37.0	39.0	24.7	18.3	20.3	5.6	8.8	5.0
Marathon	58.6	57.4	62.8	15.5	20.0	22.1	6.0	9.8	8.0
TB-234	42.3	54.2	65.3	12.2	14.2	14.2	5.2	9.5	8.8
LSD (CV or S)		6.6			4.7			2.6	
LSD (CVxS)		11.4							
Sig CV		***			*			NS	
S		*			NS			*	
CVxS		*			NS			NS	
				B					
Claudia	9.1	11.4	8.2	6.7	8.0	7.6	0.1	0.6	0.3
Marathon	9.6	12.3	14.7	5.5	9.2	11.2	1.3	3.3	2.2
TB-234	5.2	10.9	9.3	5.6	6.9	5.4	1.2	2.5	2.0
LSD (CV or S)		2.1			1.9			0.6	
LSD (CVxS)		3.7			3.4			1.0	
Sig CV		**			*			***	
S		**			*			***	
CVxS		*			*			*	

[a] Probability level: ***$P < 0.001$, **$0.001 < P < 0.01$, *$P < 0.05$, NS = not significant, 16 degrees of freedom for LSD. C = control, LS = low S, and HS = high S.

At maturity, there were no differences in dry matter between Claudia and Marathon but TB-234 had the lowest dry matter.

Changes in S Uptake and Glucoraphanin Content. *Vegetative Stage.* Glucoraphanin content of whole plant significantly increased with gypsum application in Marathon by 50 and 70% at the low and high S rates, respectively, and TB-234 at low S by 68%, whereas there were no significant differences in plant S content for all three broccoli cultivars (**Figure 1A**). The low S uptake from gypsum at this stage may be due to the form of S not being readily available for plant uptake since anhydrous gypsum is fairly insoluble in water. Gypsum was used in this study to examine the influence of S fertilizer on glucoraphanin levels because previous studies have shown that gypsum S significantly increased S uptake and glucosinolate levels in field-grown rapeseed (*15*, *18*, *19*, *25*, *26*). In addition, gypsum is a cheap source of S that can easily be applied before transplanting of the broccoli seedlings. Another reason for low S uptake by the plant may have been due to young plants having a limited capacity for S uptake and assimilation. Also, low temperatures during the vegetative stage after the first application of S (July−August) probably restricted plant S uptake.

The highest S and glucoraphanin contents were found in leaves, followed by stems and roots (**Table 2**). Leaves contained more than half of the total plant S and total glucoraphanin (**Figure 2A**), which reflected the high partitioning of dry matter to leaves at this stage (data not shown). S content was significantly increased by gypsum applications only in leaves of TB-234 (**Table 2A**). Glucoraphanin content tended to increase in response to gypsum in some plant organs and cultivars, although the low S was at least as effective as the high S (**Table 2B**). The response of leaf glucoraphanin content to applied gypsum was more pronounced than leaf S content. The S results agree with previous studies in canola (*19*, *25*), which indicated that S application (as gypsum) significantly increased leaf S content and the leaves also contained higher S than stems or roots.

Green Head Stage. Gypsum applications significantly increased plant S content in TB-234 by 32% at low S and 27% at high S and also significantly increased glucoraphanin content in TB-234 for both S rates (48% at the low S and 43% at the high S) (**Figure 1B**). Glucoraphanin content in Claudia was significantly increased (66%) but only at the high S rate. A significant increase for both S and glucoraphanin contents at this stage may have been due to (i) greater S availability after gypsum was reapplied for a second time and (ii) greater S demand from plants growing rapidly as temperatures were increasing in spring.

The highest S content was found in stems, followed by broccoli heads and leaves, whereas glucoraphanin content was the highest in broccoli heads; roots contained the lowest contents for both S and glucoraphanin (**Table 3**). In broccoli heads, S content accounted for only 34, 30, and 24% of total plant S while glucoraphanin content accounted for 63, 54, and 49% of total plant glucoraphanin in Claudia, Marathon, and TB-234, respectively (**Figure 2B**). The proportion of the total content in the heads was much greater for glucoraphanin than S content, suggesting preferential partitioning of S into glucoraphanin in the green heads.

Glucoraphanin content of green heads and roots was significantly increased by gypsum, although high S did not always cause a significant increase over low S (**Table 3B**). Glucoraphanin content of green heads was more responsive to S supply in Claudia than Marathon or TB-234. The findings of Fieldsend and Milford (*19*) found that S application significantly increased glucosinolate content and S in rapeseed in both inflorescences and vegetative parts.

Maturity. Plant S content increased significantly in Marathon by 39 (low S) and 20% (high S) and in Claudia by 30 (low S) and 33% (high S), but there was no significant increase in TB-234 (**Figure 1C**) because of reduced pod and seed set for that genotype. However, plant glucoraphanin content had a tendency to increase for all three broccoli cultivars at maturity with the strongest increase at the high S rate in Claudia (more than 100%) and TB-234 (66%), whereas Marathon had the strongest increase at the low S rate (45%). The plants at this stage were large and had developed many pods and seeds, which would have required more S uptake for glucoraphanin synthesis, particularly when considering the high glucoraphanin content of seeds. These results agree with those of Fieldsend and Milford (*19*) who reported that rapeseed S content and glucosinolate content of the whole plant significantly increased at maturity in response to 40 kg ha^{-1} of S (applied as gypsum).

The highest S content was generally found in stems and seeds, while pods and roots only contained small amounts of S (**Table 4A**). However, seed S content of TB-234 was low, because of poor seed set. Glucoraphanin content was much higher in mature seeds than other organs (**Table 4B**). The S content of mature seeds accounted for 39 (Claudia), 58 (Marathon), and 24% (TB-234) of total plant S whereas glucoraphanin content accounted for 93 (Claudia and Marathon) and 89% (TB-234) of total plant glucoraphanin with less than 6% present in stems and less than 10% in pods for all three cultivars (**Figure 2C**). Nearly all of the plant glucoraphanin was present in the seeds at maturity, implying redistribution of glucoraphanin from other organs to the seeds or glucoraphanin breakdown in the other organs followed by de novo synthesis in the seeds during seed filling.

Glucoraphanin content was increased by S supply in all plant organs with the strongest response occurring in Marathon to the low S, while Claudia and TB-234 had the strongest response to the high S (**Table 4B**). In addition, many reports have also found S fertilizers, applied as potassium sulfate, ammonium sulfate, or gypsum, to increase glucosinolate and S contents in seeds and pods of rapeseed (*15*, *17*, *19*, *25*, *26*).

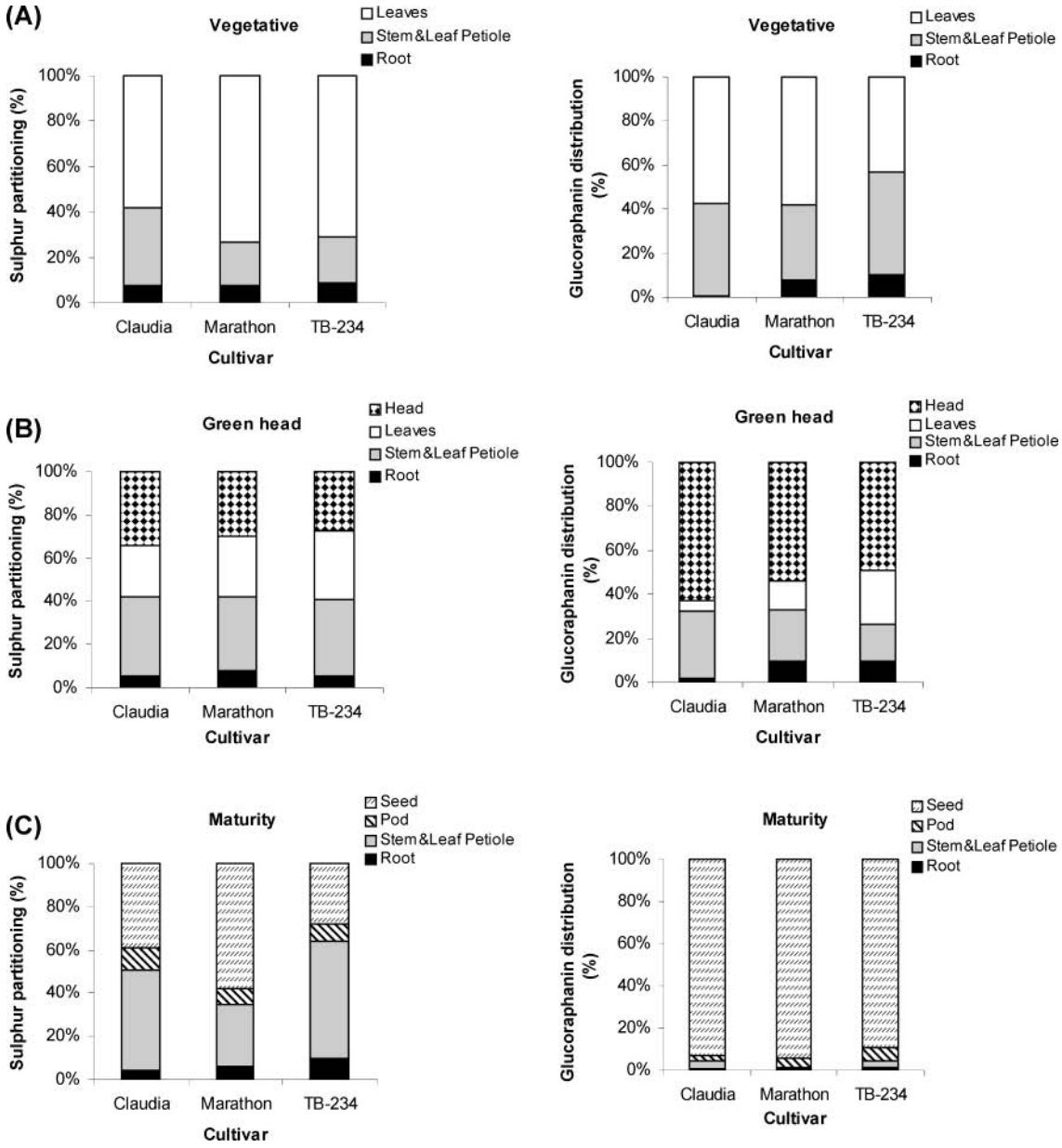

Figure 2. Partitioning of S and glucoraphanin to different plant organs (% of total plant S and glucoraphanin) of three broccoli cultivars (Claudia, Marathon, and TB-234) at different growth stages (**A**: vegetative, 88 DAS; **B**: green head, 108 or 125 DAS; and **C**: maturity, 220 or 235 DAS).

Partitioning of S into Glucoraphanin for Green Heads and Mature Seeds of Broccoli. At the green head stage, S present in glucoraphanin (glucoraphanin-S) accounted for only 4.1−10.0% of total S content in the heads (**Table 5**). In mature seeds, glucoraphanin-S accounted for only 5.3−10.2% in Claudia but accounted for much more (40.0−46.3%) in seeds of Marathon and TB-234. There were highly significant ($P < 0.01$) genotypic differences in partitioning of S into glucoraphanin. Partitioning of S into glucoraphanin also increased with gypsum applications, with the strongest increase occurring in Claudia at the high S application in both broccoli heads and mature seeds.

Fieldsend and Milford (1994) also found in rapeseed that S present in glucosinolates accounted for only 4−5% of the total S in vegetative tissues and flowers at the flowering stage (*19*). In addition, a large proportion of S (70−90%) taken up by the plant was present in rapeseed leaves as sulfate while glutathione and glucosinolates accounted for less than 1% of S (*27*).

In mature seeds, S present in glucoraphanin in the control treatment accounted for only 5% of the total S seed content in the low glucoraphanin cultivar, Claudia, but accounted for 40% in the moderate and high glucoraphanin cultivars, Marathon and TB-234 (**Table 5**). This indicated that the major form of S in mature seeds was S present in glucoraphanin. The results agree with those of Fieldsend and Milford (*19*) who indicated that in rapeseed at maturity, S in seeds accounted for only 16% of the total S with 12% of seed S in form of glucosinolate-S and this proportion of seed S increased with high S application to 30%. Their results also showed that 40% of plant S remained in vegetative tissues with less than 2% of this S present in glucosinolates.

Changes in Glucoraphanin Concentration. At the vegetative stage, glucoraphanin concentrations were low (0.10−5.32 μmol g^{-1} DW), reflecting the low S uptake of young broccoli plants in winter (**Figure 1A**). At the green head stage, glucoraphanin concentration was highest in broccoli heads (**Table 6**), as was the case for glucoraphanin content. At this stage, glucoraphanin concentrations were low (0.47−4.19 μmol g^{-1} DW) in vegetative tissues. Higher S uptake in spring (**Figure 1B**), together with preferential partitioning of S into glucoraphanin in green heads (**Figure 2B**), resulted in high glucoraphanin

Table 3. Effects of S Supply on S (A) and Glucoraphanin (B) Contents (mg Organ^{-1}) in Leaves, Stems and Leaf Petioles, Roots, and Heads of Broccoli Cultivars Claudia, Marathon, and TB-234 at the Green Head Stage (108 or 125 DAS)[a]

	leaves			stems and leaf petioles			roots			heads		
organ/cultivar	C	LS	HS	C	LS	HS	C	LS	HS	C	LS	HS
						A						
Claudia	139.4	181.7	129.9	217.4	229.1	223.8	33.9	32.3	40.8	203.2	218.5	210.6
Marathon	226.3	216.9	293.7	278.3	262.4	281.8	63.9	61.0	75.8	240.4	249.9	244.6
TB-234	363.4	529.2	476.8	394.9	437.0	433.0	65.0	112.7	109.5	308.6	419.3	417.6
LSD (CV or S)		60.3			48.1			16.2			40.6	
LSD (CVxS)		104.4						28.1			70.3	
Sig CV		***			***			***			***	
S		NS			NS			*			NS	
CVxS		*			NS			*			*	
						B						
Claudia	4.3	5.8	5.3	26.1	27.6	31.6	1.8	1.8	2.8	54.5	79.8	103.2
Marathon	19.0	12.6	22.6	34.4	37.2	44.5	13.6	16.0	22.2	78.6	86.1	98.0
TB-234	32.1	38.0	35.0	22.0	37.4	30.7	12.5	18.6	18.2	63.6	88.3	102.2
LSD (CV or S)		6.6			7.6			2.6				
LSD								4.5			37.6	
Sig CV		***			*			***			NS	
S		NS			*			**			*	
CVxS		NS			NS			*			*	

[a] Probability level: ***P < 0.001, **0.001 < P < 0.01, *P < 0.05, NS = not significant, 16 degrees of freedom for LSD. C = control, LS = low S, and HS = high S.

Table 4. Effects of S Supply on S (A) and Glucoraphanin (B) Contents (mg Organ^{-1}) in Stems and Leaf Petioles, Roots, Pods, and Seeds of Broccoli Cultivars Claudia, Marathon, and TB-234 at Maturity (220 or 235 DAS)[a]

	stems and leaf petioles			roots			pods			seeds		
organ/cultivar	C	LS	HS	C	LS	HS	C	LS	HS	C	LS	HS
						A						
Claudia	778.0	976.2	1051.6	71.1	89.6	86.9	175.6	261.7	249.9	651.7	862.8	843.7
Marathon	539.2	703.4	670.5	111.6	128.1	123.4	137.7	205.7	160.3	1082.2	1566.5	1301.9
TB-234	784.7	524.8	727.0	145.4	137.5	145.4	113.2	117.9	163.5	410.4	495.4	631.7
LSD (CV or S)		131.6			21.3			23.2			112.9	
LSD (CVxS)		227.9						40.2			195.6	
Sig CV		***			***			***			***	
S		NS			NS			***			***	
CVxS		*			NS			*			*	
						B						
Claudia	7.1	5.8	5.9	0.8	1.1	1.1	4.5	4.7	4.7	171.1	308.5	427.6
Marathon	22.1	20.2	41.2	13.4	17.0	12.5	97.7	98.6	96.3	2141.3	3152.0	2964.2
TB-234	28.9	43.1	43.6	11.6	13.4	14.4	62.3	54.7	135.8	849.4	1029.3	1384.0
LSD (CV or S)		5.0			3.7			21.3			237.5	
LSD (CVxS)		8.6						36.9			380.0	
Sig CV		***			***			***			***	
S		***			NS			*			***	
CVxS		**			NS			*			*	

[a] Probability level: ***P < 0.001, **0.001 < P < 0.01, *P < 0.05, NS = not significant, 16 degrees of freedom for LSD. C = control, LS = low S, and HS = high S.

Table 5. Partitioning of S into Glucoraphanin (Glucoraphanin-S as % of Total S in the Organ) in Broccoli Heads and Mature Seeds of the Three Broccoli Cultivars Claudia, Marathon, and TB-234 as Affected by S Application[a]

	heads			seeds		
organ/cultivar	C	LS	HS	C	LS	HS
Claudia	5.2	7.8	10.0	5.3	7.2	10.2
Marathon	6.6	7.0	8.1	40.0	40.6	46.3
TB-234	4.1	4.3	5.0	41.6	42.0	44.3
LSD (CV or S)		1.9			3.4	
LSD (CVxS)		3.2			5.8	
Sig CV		**			**	
S		*			*	
CVxS		*			*	

[a] Probability level: **0.001 < P < 0.01, *P < 0.05, 16 degrees of freedom for LSD. C = control, LS = low S, and HS = high S.

concentrations in green heads. Gypsum applications significantly increased glucoraphanin concentrations in green heads, particularly in Claudia (**Table 6**). This was mainly as a result of greater partitioning of S into glucoraphanin (**Table 5**), rather than higher S uptake.

At maturity, glucoraphanin concentration was very high in seeds, particularly in Marathon and TB-234 (**Table 6**). This resulted from high S uptake from green head stage to maturity (**Figure 1B**,**C**), redistribution of S from vegetative tissues to the seeds (**Figure 2B**,**C**), and high partitioning of S into glucoraphanin in seeds of Marathon and TB-234 (**Table 5**). Glucoraphanin concentrations were low in stems (0.13−1.31 μmol g^{-1} DW) and roots (0.14−1.69 μmol g^{-1} DW) but higher in pods, at least for Marathon and TB-234 (**Table 6**). Gypsum applications significantly (P < 0.001) increased glucoraphanin concentration in mature seeds, particularly at the high S rate. This was as a result of greater S uptake for Claudia and Marathon but not TB-234 (**Figure 1C**) and greater partitioning of S into glucoraphanin (**Table 5**). Hocking et al. (*20*) also reported that total glucosinolate concentrations in seeds of *B. napus*, which received the high S application, were higher than those in seeds of the low S, and delaying S topdressing (as potassium sulfate) until flowering resulted in a significant increase in seed glucosinolate concentration as compared to S application at sowing. However, the present study determined

Table 6. Effect of S Supply on Glucoraphanin Concentration in Green Heads (108 or 125 DAS) and in Mature Pods and Seeds (220 or 235 DAS) of Broccoli Cultivars Claudia, Marathon, and TB-234[a]

	green heads			mature pods			mature seeds		
organ/cultivar	C	LS	HS	C	LS	HS	C	LS	HS
Claudia	6.60	10.57	14.54	0.35	0.32	0.24	10.42	14.64	20.81
Marathon	8.80	9.59	11.63	6.26	4.08	5.29	120.05	129.79	148.96
TB-234	6.69	6.80	8.17	6.49	5.31	10.31	218.57	207.85	227.01
LSD (CV or S)		2.47			0.88			7.27	
LSD (CVxS)					1.53				
Sig CV		*			***			***	
S		*			***			***	
CVxS		NS			***			NS	

[a] Probability level: ***P < 0.001, *P < 0.05, NS = not significant, 16 degrees of freedom for LSD. C = control, LS = low S, and HS = high S.

the effect of S fertilizer only on glucoraphanin, the precursor of anticancer sulforaphane, since other glucosinolate standards were not commercially available. Therefore, it is also important to examine the effect of S fertilizer on other individual glucosinolates and total glucosinolates present in the broccoli.

CONCLUSIONS

S fertilizer (gypsum) increased total S and glucoraphanin accumulation in the three broccoli cultivars during plant development. Increasing S content with gypsum applications led to an increase in glucoraphanin content in all plant organs. A large increase was found in broccoli heads and mature seeds. Plant S uptake was initially slow, probably due to reduced S availability from the applied fertilizer, and a slow rate of S uptake by the young plants. During the later vegetative stage, S uptake increased as growth accelerated with warmer spring temperatures, and glucoraphanin production increased. During the early growth stages, the cultivars that contained moderate to high concentrations of glucoraphanin in seeds, Marathon and TB-234, were highly affected by both gypsum applications with an increase in total S and glucoraphanin contents in most of plant organs, but these effects were small in the low glucoraphanin cultivar, Claudia. The young plants of the high glucoraphanin cultivars may have a greater transport and assimilatory capacity for S and the subsequent incorporation into glucoraphanin. However, at maturity, gypsum applications increased both total S and glucoraphanin contents in all three broccoli cultivars as these crucifers have a high S demand for glucoraphanin synthesis in the seeds. In nearly all plants, glucoraphanin was present in the seeds at maturity, implying redistribution of glucoraphanin from other organs to the seeds or glucoraphanin breakdown in the other organs followed by de novo synthesis in the seeds during seed filling.

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